Chapter 10
Cytokines as Active Factors in Focal Segmental Glomerulosclerosis

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Abstract Cytokines may play important roles in primary focal segmental sclerosis. One or more may be the elusive circulating factor(s) that induces proteinuria in this condition, and their local renal tissue production may be crucial to the development of glomerular and interstitial fibrosis.

To this date, the data on a specific laboratory test that will allow us to detect the presence of a circulating factor are controversial. Moreover, only three cytokines (CLC-1, VEGF, and TGF alpha) have been suggested as putative circulating factor. However, there is no conclusive evidence of the presence of these cytokines in the serum of focal segmental glomerulosclerosis patients, and no proteinuria has been consistently observed when the experimental animal has been infused with these cytokines.

There is more compelling evidence for the role of an increased production of TGF beta in the development of glomerular and interstitial fibrosis in this condition. The increased TGF beta observed is produced at the level of the glomerulus and interstitium triggered by an unknown mechanism.

Keywords Focal segmental glomerulosclerosis • Cytokines • Circulating factor

10.1 Definition

Focal segmental glomerulosclerosis (FSGS) refers to a phenotype of glomerular injury induced by a number of clinic-pathological syndromes and characterized only by sclerotic lesions involving less than 50 % of glomeruli (focal) and less than 50 % of the glomerular tuft (segmental) [1].

FSGS represents the second most common type of nephrotic syndrome in children, accounting for about 8 % of cases of children with nephrotic syndrome

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who undergo biopsy [2] but remains the leading glomerular disease causing end-stage renal disease in both pediatric and adult populations [3].

10.2 Classification

Primary or idiopathic FSGS, accounting for 80 % of FSGS cases, is considered when no underlying cause for the disease process is identified [4]. The causative mechanism(s) of primary FSGS has remained elusive, although there is robust clinical evidence supporting the presence of a circulating factor, yet to be determined [5, 6]. This review will focus on the role of cytokines (1) as presumptive circulating factor(s) thought to induce proteinuria in FSGS and (2) factors involved in the glomerular and tubular interstitial sclerosis in this disease. The role of other proposed circulating factor in primary FSGS such as suPAR will be reviewed in Chap. 9.

10.3 Cytokine(s) as a Circulating Factor in the Pathogenesis of Proteinuria in Primary FSGS

10.3.1 Clinical Evidence for the Presence of Circulating Factor that Induces Proteinuria in Primary FSGS

In 1972, Hoyer observed recurrence of the nephrotic syndrome in 3 patients [5] with primary FSGS immediately after kidney transplantation and suggested that proteinuria could be due to a circulating factor. This observation has been confirmed, and currently primary FSGS has shown to recur in about 40 % of patients after the first kidney transplantation [7]. The histological findings of recurrent FSGS in the transplanted kidney are similar to those observed in the native kidney, from minimal, if any, morphological changes on light microscopy and extensive podocyte foot processes effacement in early stages to patchy sclerotic glomerular lesions in advance stages.

Further clinical evidence supporting the presence of a circulating factor is provided by the reduction of proteinuria with plasmapheresis [8–13] and immunoadsorption [14–17] in those patients with FSGS recurrence after transplantation and the reported transference of the putative factor from mother who had primary FSGS to fetus causing transitory proteinuria in the newborn [18]. Finally, in 2012, Gallon et al. [19] observed resolution of the proteinuria and normalization of glomerular filtration after re-transplantation of a kidney from a patient with primary FSGS who experienced recurrence after transplantation to a patient with no underlying glomerular disease.

10.3.2 Experimental Model of Primary FSGS and Circulating Factor(s)

In 1983, Kato showed that the Buffalo/Mna rats spontaneously experience selective proteinuria by 10 weeks of age, with podocyte effacement at 2 months and sclerotic lesions by 4 months of age [20].

In 2002, Le Berre et al. [21] suggested that proteinuria and histological changes found in the Buffalo/Mna were associated with the presence of a circulating factor. He observed that kidneys from healthy LEW.1 W rats developed proteinuria and podocyte foot process effacement after transplantation into proteinuric Buffalo/Mna rat, but no such changes were observed when grafted into healthy Wistar Furth rats. In this study, recurrence of proteinuria (>0.2 g/mmol) occurred 20 days after transplantation. None of the transplanted Buffalo/Mna rats experienced immediate recurrence as often observed in primary FSGS patients. However, supporting the concept of a circulating factor, proteinuria and glomerular lesions of the Buffalo/Mna kidney regressed after kidneys from these proteinuric rats were transplanted into healthy LEW.1 W rats. Unfortunately, Le Berre's results remain to be validated by other groups**.** Thus, the concept of the Buffalo/Mna rat as a model of primary FSGS remains to be determined.

10.3.3 Infusion of Serum or Fraction of Serum from FSGS Patients in Animals

10.3.3.1 Infusion of Serum from FSGS Patients

Zimmerman [22] in 1980 found that rats infused intravenously for 90 minutes with serum from 1 patient with recurrent FSGS after transplantation demonstrated a marked increase in proteinuria compared to those rats administered with sera from 10 patients with other proteinuric glomerulopathies (membranoproliferative glomerulonephritis, membranous nephropathy, minimal change disease, and lupus nephritis) and 1 patient with FSGS without recurrence after transplantation. A marked increase in albuminuria was documented during the infusion period and 60 min afterwards. At the end of the infusion, albuminuria represented 53 % of the total protein excreted. Albuminuria increased from baseline of a mean of 250–1340 μg/h at the end of the infusion. However, the response was rather variable (albuminuria $1340 + -2548$ mcg/h, mean $\pm$ SD) demonstrating that not all the rats responded with massive proteinuria. If a circulating factor is responsible of the recurrence of nephrotic syndrome after transplantation, one may assume that such circulating factor would be also present in patients with primary FSGS nephrotic syndrome prior to transplantation. In the same study, rats infused with serum from a FSGS patient presenting with massive proteinuria prior to transplantation

experienced similar urinary protein excretion than those observed in control rats infused with saline.

Avila-Casado [23] studied the effect of serum infusion (1 mL, once daily, intravenously for 5 days) from 10 patients with collapsing FSGS and 10 patients with FSGS not otherwise specified (NOS) variant in rats. Proteinuria significantly increased from baseline as soon as 24 h after the infusion and kept rising for the next 5 days only in rats injected with sera with collapsing FSGS but not in those rats infused with FSGS NOS variant. Avila-Casado repeated the experiments but injecting only isolated IgG and serum without IgG. The injection of these two components of serum from collapsing FSGS patients also resulted in proteinuria but less than that observed with whole serum. Authors suggested the possibility of the presence of more than one causative factor in collapsing FSGS.

10.3.3.2 Infusion of Plasma Fractions from FSGS Patients

Recurrence of FSGS after transplantation often improves or resolves after plasmapheresis or protein A immunoadsorption [8–17]. These observations have led to the infusion of eluates from plasmapheresis from patients with FSGS recurrence after transplantation to rats to assess for the presence of the circulating factor in the eluates.

Savin's group [24] collected plasma during plasmapheresis from 4 FSGS patients who experienced recurrence after kidney transplantation and from pooled healthy individuals. In an attempt to isolate the "active" fraction, plasma was purified in a multistep process and concentrated as 70 % supernatants. Rats (3–8 per patient's sample) infused intravenously with 1 ml of 70 % FSGS supernatants (12 mg of protein) experienced a significant increase in proteinuria from 6 to 24 h after infusion, whereas no such increase in proteinuria was observed in rats infused with 70 % supernatants from pooled control subject. It is unclear if proteinuria was due to albuminuria.

Three years later, Savin's group [25] replicated results from their original work [24]. Rats infused with 70 % supernatants from 4 patients with FSGS recurrence, but not those receiving 70 % supernatants from pooled control subjects, developed proteinuria 6–24 h after infusion. Of interest, the mean albuminuria at 24 h (peak proteinuria) was 700 μg/mg creatinine; however, the mean peak proteinuria at that time was 9.4 mg/mg creatinine, demonstrating that albumin represented only a small percentage (0.07 %) of the total urinary protein excreted in these rats.

Dantal et al. [26] collected plasma from 8 patients who presented nephrotic syndrome after transplantation (5 FSGS, 1 IgA nephropathy, 1 diabetes nephropathy, and 1 nephroangiosclerosis). In the 5 FSGS patients with recurrence of the nephrotic syndrome, plasma was collected after plasmapheresis. Plasma was purified by a similar approach to that described by Savin. The 70 % supernatant fraction was filtered using 50- and 30-kD membranes. The resultant supernatants contained three proteins: albumin, apolipoprotein AI, and a 43-kD protein identified as

orosomucoid. This 43-kD molecule was found at a much higher concentration in the FSGS samples than in non-FSGS patients.

Dantal [26] infused 70 % supernatant from 5 FSGS patients into rats. Each rat received one sample of individual fraction (from 1 patient), and this sample was injected into at least three rats (from three to seven rats for one individual sample in one type of injection). Rats were injected directly into the aorta with 1 ml of the 70 % FSGS supernatants. The infusion lasted 5–8 min. Rats developed significant, but transient, proteinuria 24 h after infusion. Similar rise in proteinuria was observed in 19 rats infused with 70 % supernatants from four healthy individuals and ten rats infused with a similar volume of isotonic saline. However, no increase in urinary protein excretion was observed when 20 rats were infused with 70 % supernatants from 3 non-FSGS patients. In their experiments, albumin accounts for 76 % of the total urinary protein in the rats, and the proportion remained unchanged after supernatants.

In a subsequent experiment [26], rats were infused with 1 ml of 70 % supernatants from 5 FSGS, 3 non-FSGS patients, and 3 healthy controls at 0, 48, and 72 h. The supernatant was infused either intravenously (7 rats) or intraperitoneally (12 rats). No increase in proteinuria was observed among the groups. In addition, protein A eluted from immunoadsorption column from 1 patient with FSGS recurrence and 1 nephrotic non-FSGS patient was infused intra-arterially to 7 rats by Dantal. Proteinuria was not significantly different among the two groups neither before nor after infusion. Finally, Dantal purified orosomucoid from plasma of 3 recurrence FSGS posttransplantation patients, 1 non-FSGS nephrotic patient, and 1 healthy control. Rats ($n = 7$ per group) infused with these preparations experienced a significant increase in proteinuria 24 h later, but no differences were observed among the three groups.

In summary, two research groups infused similar plasma fractions collected from plasmapheresis from patients with FSGS recurrence into rats with opposing results. Savin's group [24, 25] demonstrated increase in proteinuria only in those rats infused with FSGS recurrence supernatants. Dantal did not [26]. These contrasting results do not have a readily explanation. In addition, albuminuria observed in Savin study does not mimic what it is observed in FSGS patients and therefore questions its clinical significance.

10.3.4 Is There a Laboratory Test that Allows Us to Detect the Presence of a Pathogenic Circulating Factor in Patients with FSGS?

Savin et al. [27] in 1992 developed an in vitro technique that, according to these authors, allowed detection of a circulating factor that increases the glomerular permeability to plasma proteins in FSGS patients.

Savin, using rat isolated glomeruli, observed that changes in glomerular size were directly proportional to oncotic gradient conditions in the media. Glomerular volume in hypotonic media will increase. In the presence of an altered permeability barrier, presumably due to the FSGS circulating factor, proteins would escape from the intra-glomerular tuft, decreasing the capillary osmotic pressure and, therefore, curtailing the increase in glomerular volume induced by hypo-osmotic media. In contrast, control serum would not interfere with the increase in glomerular volume after hypo-osmotic media.

Savin's protocol is as follows: rat glomeruli free of Bowman's capsule are isolated from the renal cortex of Sprague–Dawley rats and initially incubated with 4 g/dl bovine serum albumin (BSA). After a 10-min incubation with a 1:50 dilution of serum or plasma fractions from FSGS patients, patients with other renal diseases and control subjects, medium is replaced with 1 g/dl BSA to generate an oncotic gradient. Volume changes in rat glomeruli are determined by video recording each glomerulus individually before and after the media exchange. Then, glomeruli are measured with a ruler on a magnified image of the glomerulus in a screen, and diameter (D) is defined as the average of 4 diameters at 45° – degree angles to each one. The glomerular volume is derived from the equation $V = 4/3\ \pi\ (D/2)3$. Finally, glomerular permeability to albumin is calculated as P Alb = 1 – (Δ volume experimental glomerulus/Δ volume control – nonexposed to patient sera/plasma) [9, 27]. Δ Volume is calculated as: (final volume-initial volume)/initial volume. P Alb ranges from 0 (representing glomeruli with no capillary leak) to 1 (glomeruli with maximal capillary leakage).

Isolated glomeruli incubated with sera from patients with FSGS recurrence demonstrated a significantly higher P Alb than those incubated with sera from FSGS patients without recurrence after transplantation, patients with renal diseases other than FSGS, and control subjects [9]. Savin found that P Alb, using pretransplant sera, was greater than 0.5 in 6 of 7 patients who experienced FSGS recurrence after transplantation. In contrast, P Alb was less than 0.5 in 19 of 23 patients without FSGS recurrence. Thus, a P Alb value > 0.5, using pretransplant sera, was suggested as a risk factor for FSGS recurrence after transplantation [9].

Dall'Amico et al.[28], using the same in vitro technique described by Savin, found that, using pretransplant sera, FSGS recurred posttransplantation in 11 of 13 children with elevated in vitro P Alb (>0.6), while 4 of 12 patients with P Alb <0.6 did not have recurrence of FSGS. Twelve out of 25 children had a negative in vitro test (P Alb <0.6) before transplantation despite the presence of active nephrotic syndrome. These results are similar to those described by Savin [9].

If P Alb reflects the presence of a circulating factor that induces proteinuria, one may assume that P Alb would be increased (>0.5) in all patients with FSGS and massive proteinuria in native or transplanted kidney. However, 35 out of 55 patients (64 %) with active FSGS, included in Savin and Dall'Amico studies [9, 28], had a negative in vitro P Alb before kidney transplantation. Moreover, none of the groups of patients with FSGS studied by Savin (non-transplanted and transplanted patients with and without FSGS recurrence) presented a mean P Alb >0.5 [9].

Two groups have also found P Alb >0.5 in patients with primary FSGS and nephrotic syndrome [29, 30]. However, all their patient's samples were tested at Savin's laboratory. Therefore, none of these studies independently assess the validity of Savin's method to evaluate for the presence of the circulating factor.

Godfrin et al. [31] used the same principles of Savin's to develop an in vitro test to detect the presence of a circulating factor. However, they measured the glomerular volume using a multi-sizer coulter instead of videomicroscopy. This allowed to measure a larger number of glomeruli (1500 per experiment counted over a period of 1 min), overcoming one of the weaknesses of the Savin's method. In addition, Godfrin compared the glomerular volume of glomeruli exposed to controls sera using a different set of glomeruli than those exposed to serum from FSGS patients, whereas Savin compared the changes in volume of the same glomeruli before and after the exposure to FSGS or control serum.

Glomerular volume variation (GVV) was estimated by Godfrin as: 1-volume of experimental glomeruli/volume of control glomeruli, which in contrast to P Alb defined by Savin [27], did not range from 0 to 1. GVV represented the mean of three independent experiments using glomeruli from three different animals per serum sample. In addition, Godfrin incubated glomeruli with patients or controls serum for 16 min instead of Savin's 10. In addition, the oncotic gradient was generated by changing BSA concentration from 6 % to 1 % compared to 4 % to 1 % used in Savin studies [27].

Godfrin et al. [32] studied the effects of pretransplant sera from 80 FSGS and end-stage renal disease. All these patients underwent renal transplantation. Fifty-four had recurrence of FSGS. Patients' GVV was significantly higher in FSGS prior to transplantation compared to 19 patients with ESRD due to PKD, 18 patients with uropathies, 26 patients with membranous nephropathy, and 10 healthy controls. GVV was elevated in 28 FSGS patients who underwent renal transplantation. GVV remained elevated in 14 patients who experienced recurrence, whereas there was a significant decrease after transplantation in 14 who did not recur. Thus, in contrast to Savin's results [9], this in vitro assay, using pretransplant sera, failed to predict recurrence after transplantation.

Savin's search for circulating factor(s) has focused on the study of plasma fractions of those patients with FSGS and recurrence of the nephrotic syndrome posttransplantation. She claims that any fraction with a P Alb > 0.5 should contain the circulating factor [9].

The isolation of this presumptive circulating factor had involved a laborious multistep process [9, 24, 33, 34]. Plasma was collected after plasmapheresis. In a first step, lipoproteins and chylomicrons were removed. As the P Alb of the remaining supernatant was >0.5, it was thought that the circulating factor was a protein. This was supported by fact that P Alb activity >0.5 was suppressed with the addition of protease or by heat protein denaturation. In the next step, proteins in the supernatant were precipitated at stepwise concentrations of ammonium sulfate. Only the 70 % and 80 % supernatant fractions containing a minimal amount of proteins (1.8 % and 1.4 % of total plasma protein, respectively) showed a P Alb

>0.5 [9]. A subsequent study by the same group found no P Alb >0.5 in the 80 % supernatant fraction although P Alb was still > 0.5 in the 70 % fraction [24].

Using exclusion chromatography, Savin showed that fraction with a P Alb activity >0.5 was associated with a molecular weight between 50 and 100 kDa and an anionic charge at pH of 6.0 [9]. In a follow-up report, centrifugation-based membrane ultrafiltration of the 70 % supernatant suggested that the activity >0.5 was recovered from 30 to 50 kD fraction [24]. However, when authors used affinity chromatography to isolate the factor, they found that P Alb >0.5 was present in a fraction with a molecular weight < 30 kD [35]. The difference in molecular weight was attributed to a possibly degradation of the active substance during purification or aggregation of molecules in the whole plasma.

Infusion of "active" (P Alb > 0.5) plasma fractions (30–50 kD) from FSGS patients into rats led to proteinuria in Savin's studies [24, 25]; however, these results could not be replicated by Dantal's group [26].

10.3.4.1 Do P Alb or GGV Results Reflect the Presence or Absence of a Circulating Factor in FSGS Patients?

The test has some issues that raise questions as its reliability as a tool to detect the presence of a circulating factor in FSGS:

1. Lack of specificity for active primary FSGS: P Alb > 0.5, reflecting an impaired glomerular filtration barrier due to a presumed circulating factor, is present under experimental conditions [27, 36–39] or diseases not known to be caused by a circulating factor [40–42]. P Alb >0.5 is present when glomeruli are incubated with TNF alpha [36], superoxide [37], antibodies to protein tyrosine phosphatase receptor [38], and beta1 integrin [39]. Furthermore, two studies reported an elevated P Alb in patients known to have proteinuria linked to nephrin or podocin mutation [41, 42]. Similarly, increased GVV is not exclusive of FSGS patients. GVV is higher in patients with end-stage renal disease (ESRD) regardless the underlying etiology compared to that observed with healthy donors [26].
2. Reliability: Savin stated that the reliability of her assay was high based on two findings: (1) the correlation among multiple determinations of glomerular permeability to albumin with the use of serum from 35 patients was 0.72 ($P < 0.001$), and (2) repeated measures of P Alb in the 35 patients varied less than 0.3 in 83 % of cases [9]. Thus, a variation of 0.3 or less was not considered as significant. However, considering that the proposed P Alb ranges from 0 to 1, a variation of 0.3 would mean that a repeat experiment can differ by 30 % compared to the initial result. Furthermore, it is unclear if P Alb determined in four or five glomeruli is representative of the ongoing process in the remaining glomeruli.
3. No correlation between P Alb or GVV and proteinuria [29, 32, 41–43]: As previously mentioned, most of nephrotic FSGS patients had a negative assay

at pretransplantation [9, 28]. Moreover, the P Alb failed to predict occurrence of FSGS in patients initially diagnosed with idiopathic nephrotic syndrome or to predict the progression to renal failure or short-term response to prednisone [29].

Thus, it has not been proven with certainty that Savin's or Godfrin's in vitro assays using isolated glomeruli reflect the presence or absence of a circulating factor in FSGS patients. The theoretical background to use these assays is not supported by their results showing lack of specificity, contrasting results between techniques, and the absence of correlation with proteinuria.

10.3.5 Cytokines as Pathogenetic Circulating Factor in FSGS

10.3.5.1 Cardiotrophin-Like Cytokine-1 (CLC-1)

Isolation and Characterization of CLC-1 as Permeability Factor in FSGS

As previously mentioned, the observation that plasmapheresis [8–13] and immunoadsorption [14–17] reduce proteinuria in recurrent FSGS supports the hypothesis of a circulating factor as cause of recurrence.

Savin isolated the fraction of plasma with P Alb >0.5 in 13 patients with FSGS in a one-step process by means of galactose affinity chromatography [35]. The eluate showed P Alb > 0.5 only in the fraction <30 kD. In this fraction, cardiotrophin-like cytokine-1, a member of the IL-6 family, was the only cytokine recovered. It was found at a concentration 100 times higher in FSGS compared to control subjects. Furthermore, the addition of galactose to glomeruli incubated with serum from FSGS patients and a P Alb > 0.5 reduced the P Alb activity which could be reverted by removing galactose. Moreover, an increase of the P Alb activity was prevented by incubating glomeruli with galactose prior to treatment with serum from FSGS [35]. Savin suggested a link between galactose and CLC-1 and proposed the use of galactose to treat nephrotic syndrome in FSGS patients.

CLC-1: Mechanism(s) of Proteinuria

The mechanism(s) by which CLC-1 causes proteinuria remains to be determined. As CLC-1 has shown a high affinity for galactose and also carries P Alb >0.5, Savin hypothesized that CLC-1 may have galactose-binding sites by which it interacts with proteins of the podocyte glycocalyx and activate signal transduction in podocytes [35]. Savin also observed that effect of FSGS sera on P Alb was blocked by a monoclonal antibody against CLC-1, suggesting that the permeability defect observed in the in vitro assay was CLC-1 mediated. Moreover, CLC-1 was found to decrease the nephrin expression in cultured podocytes and glomeruli (abstract

format). However, the presumed "pathogenic" role of CLC-1 on podocytes remains yet to be established. Unfortunately, Savin et al. have not infused the CLC-1 cytokine nor the serum fraction containing CLC-1 in the experimental animal which will confirm, if proteinuria develops in these animals, its role in the pathogenesis of the proteinuria. Therefore, the clinical significance of these experimental findings needs further investigation.

CLC-1 and Galactose Therapy

The rationale to use galactose supplementation in primary FSGS is based, as previously mentioned, on the hypothesis that circulating CLC-1 interacts with podocytes through its galactose-binding sites. Thus, Savin postulated that the addition of free galactose may bind to the circulating CLC-1 forming complexes, later cleared by glucose-specific receptors in the liver, preventing its binding to podocytes resulting in a reduction of proteinuria [35]. The use of intravenous/oral galactose in FSGS was first reported by these authors in 2008 in an FSGS patient with recurrence after transplantation [35]. Despite galactose therapy, proteinuria remained unchanged and the patient progressed to end-stage renal disease. The lack of clinical response to galactose was attributed to a late onset of therapy as the patient probably had already suffered irreversible glomerular damage. However, authors were encouraged by the fact that P Alb activity from patients' plasma was reduced from 0.88 to 0 after intravenous galactose infusion.

Subsequently, galactose therapy has been reported by others to be beneficial in three case reports [44–46] and two small case series [43, 47]. However, the effect of concomitant immunosuppressive therapy or plasmapheresis could not be ruled out in those studies. In addition, half of those patients remained or achieved partial remission at best scenario. The largest study using oral galactose was published in 2013[43]. Sgambat found that a 16-week trial with oral galactose failed to reduce proteinuria in seven children with steroid resistant nephrotic syndrome (four FSGS, two FSGS recurrence after transplantation, and one minimal change disease – MCD) and eGFR > 60 ml/min/1.73 m^2. Proteinuria persisted despite a significant reduction of P Alb (0.69 vs. 0.35, p 0.009) [43].

The clinical benefit of galactose therapy in primary FSGS remains to be determined. The validity of the P Alb test to monitor galactose therapy is unclear given the lack of correlation between the test and proteinuria.

10.3.5.2 Vascular Endothelial Growth Factors (VEGFs)

The vascular endothelial growth factors (VEGFs) are dimeric glycoproteins of approximately 40 kD. Six major isoforms of VEGFs are expressed in humans, 121, 145, 165, 183, 189, and 206 amino acids [48, 49]. VEFG is constitutively expressed in podocytes and tubular-collecting and mesangial cells under certain circumstances, whereas VEGF receptors 1 and 2 are mainly expressed on

glomerular endothelial and mesangial cells, suggesting a paracrine role within the kidney [50–52].

The function of the different VEGF members is determined by their binding to VEGF receptors (VEGFRs 1, 2, and 3), of which hypoxia is the key regulator. VEGF plays a critical role in the process of vasculogenesis and angiogenesis.

VEGF Administration in Animals

VEGF has been suggested as a/the putative permeability factor in primary FSGS. To address the question whether circulating VEGF is a causative factor of proteinuria, researchers have studied the effect of injected recombinant VEGF in rats.

Iijima reported in 1992 an increased urine albumin excretion in rats after intravenous VEGF bolus infusion (50 and 100 mcg) into the tail vein. Lower VEGF doses had no effect on albuminuria. However, their results have been published only on abstract format, and, therefore, the details of the study are not available (Iijima et al. J Am Soc Nephrol. 1992;3:514).

Kankle [53] administered to Sprague–Dawley rats isolated perfused kidney 1 mcg of recombinant VEGF. Preparation was perfused for 120 min and urine was collected. Authors found that VEGF resulted in relaxation of the renal vascular bed mediated by nitric oxide, but they did not observe proteinuria.

In Sprague–Dawley rats, Webb [54] infused intravenously (0.5 ml over 10 min) 50 mcg of recombinant VEGF 165. Urine was collected for 1 h. When compared with control rats that have received normal saline, rats infused with VEGF experienced a decrease in blood pressure, urine output, and urinary protein excretion during the first hour after infusion. In VEGF-infused rats, VEGF plasma levels were above the detection assay cutoff (20 ng/ml), whereas they were below the assay threshold in control rats.

In contrast with previous studies in which VEGF was acutely administered, Garin et al. [55] infused recombinant VEGF 165 in the left renal artery of rats for 5 days at the rate of 20 or 40 ng/h using an osmotic pump device. They observed no differences in urinary protein excretion among rats receiving VEGF and the 1 % BSA control. Serum VEGF levels were not detectable by the end of the study suggesting either an increased catabolic rate or a level below the detection limit for their assay.

Above studies do not support a role of circulating VEGF in inducing proteinuria. Iijima's study cannot be given full consideration given the lack of details. It is unclear whether a longer exposure or a higher dose of VEGF may cause proteinuria.

Transgenic Models of VEGF

The advances in molecular biology have allowed the use of genetically modified animals to investigate the role of specific proteins. Eremina [56] developed three lines of transgenic mice to determine the role of VEGF in the kidney. Mice null for

podocyte VEGF-A died within 18 h of birth. Podocyte-specific heterozygosis for VEGF-A, which expresses less than normal controls, resulted in endothelial cell swelling (endothelosis) by 2.5 weeks of life. There was loss of podocyte foot processes, hematuria, nephrotic syndrome, and end-stage renal disease by 9–12 weeks. Finally, transgenic mice with podocyte overexpression of VEGF-A (isoform 164) developed proteinuria by 5 days of age associated with collapsing glomerulopathy. Authors suggested that a tight regulation of podocyte VEGF is required to preserve a normal glomerular filtration barrier since VEGF down- or upregulation led to proteinuria and severe histological changes.

Liu [57] generated transgenic rabbits with a higher expression of human VEGF in the liver and glomeruli. Serum VEGF levels were not different than those observed in control rabbits. At 8 weeks, transgenic mice experienced glomerular hypertrophy with an increased number of endothelial and mesangial cells reaching a peak at 20 weeks with the formation of microaneurysm and then focal/global sclerosis at 55 weeks. By 12 weeks, transgenic rabbits presented proteinuria compared to control. By 20 weeks, the number and size of podocytes were increased with effacement of foot processes. Authors concluded that increased expression of VEGF in glomeruli caused proteinuria.

Veron [58] observed that adult transgenic mice overexpressing podocyte VEGF-164 induced by doxycycline presented proteinuria at 1 month, glomerulomegaly, and podocyte effacement. VEGF plasma levels were not different than that seen in control rabbits. Authors suggested a causative relationship between VEGF and proteinuria since discontinuation of the VEGF164 inductor (doxycycline) resulted in resolution of proteinuria. Authors also demonstrated that podocytes expressed VEGF receptor 2, suggesting an autocrine role of VEGF which may regulate podocyte function through modulation of nephrin phosphorylation.

These studies suggest a link between podocyte VEFG and proteinuria. However, these studies showed no role for circulating VEGF in the pathogenesis of proteinuria.

VEGF in Focal Segmental Glomerulosclerosis

Podocyte VEGF in FSGS

VEGF is constitutively expressed in normal human podocytes. Immunochemistry studies have shown conflicting results regarding VEGF podocyte expression in FSGS. Ostalska-Nowicka [59] found that podocyte VEGF expression was increased in 5 FSGS patients when compared to controls. Unfortunately, authors did not provide data on proteinuria. In contrast, Noguchi [60] described 3 FSGS patients with VEGF podocyte expression similar to the one observed in normal control glomeruli.

The presence of an increased podocyte VEGF in FSGS could only support a role as a local mediator of proteinuria rather than as VEGF as a pathogenic circulating factor.

Circulating VEGF in FSGS

Data on serum VEGF in FSGS are scarce, included among other types of glomerulopathies, and hampered by the lack of association between proteinuria and serum VEGF. Cheong [61] studied VEGF in plasma of 43 children with idiopathic nephrotic syndrome. The underlying glomerular pathology was only known in 9 MCD and 8 FSGS patients. There was no significant difference in VEGF plasma levels between patients with FSGS and MCD during relapse. However, plasma VEGF was significantly increased in relapse when compared to patient in remission suggesting that plasma VEGF may be increased in FSGS patients. Unfortunately, no control group was included.

Similarly, Webb [54] measured VEGF in plasma of patients with nephrotic syndrome (22 in relapse and 13 in remission) and control subjects. No statistical differences were observed in VEGF's plasma levels or urinary VEGF/creatinine ratio among the three groups. Unfortunately, it is unknown how many patients did have FSGS because authors did not report the underlying glomerulopathy in these patients.

Therefore, currently available data do not support increase of circulating VEGF in FSGS.

10.3.5.3 Tumor Necrosis Factor-α (TNF-α)

TNF-α is a 26-kDa pro-inflammatory cytokine produced by activated macrophages, monocytes, B and T lymphocytes, natural killer cells, astrocytes, adipocytes, and glomerular mesangial and tubular cells [62, 63]. TNF-α is usually undetectable in serum of healthy humans, whereas elevated serum TNF-α levels have been associated with autoimmune disorders, sepsis, and malignancies [64–66].

In experimental models of immune-complex glomerulonephritis [67, 68] and sepsis [69], TNF-α mediates glomerular injury and proteinuria. Of interest for our discussion, TNF-α renal expression is increased in Mna/Buffalo rats prior to the onset of proteinuria [70].

Proteinuria and TNF-α: Experimental Studies

Ten rabbits were infused human recombinant TNF-α intravenously (8 mcg/kg/h at rate of 1 ml/h for 5 h) using an osmotic pump [71]. Five animals were killed at 15 and 5 at 24 h following infusion. Urinary protein excretion at 15 and 24 h was not different than the one observed prior to TNF-α infusion. Although plasma TNF-α levels were not measured in this set of experiments, authors found that administration of TNF-α, as previously described, in a different set of five rabbits led to higher plasma TNF-α levels at the end of the infusion when compared to prior to infusion (mean 283 ng/ml vs <0.04 ng/ml, respectively). Polymorphonuclear infiltration, endothelial swelling, and loss of fenestrae were found in kidneys of rabbits killed

after completion of the 5 h TNF infusion. However, no glomerular changes were observed in rabbits examined at 24 h (19 h after TNF infusion).

Garin [55] infused TNF-α through a pump at the rate of 10 or 20 ng/h into the left renal artery of Sprague–Dawley rats for 5 days. Only those rats infused with 20 ng/h dose of TNF-α showed a significant increase in proteinuria compared to baseline and controls during the last 2 days of TNF-α infusion. Serum TNF-α was higher in rats infused with 20 ng/h compared to those receiving 10 ng/h though it did not reach statistical significance. However, both groups had at least twofold TNF-α levels of that observed in MCD patients in relapse.

A role of TNF-α as a circulating factor inducing proteinuria cannot be concluded based on these experiments. Proteinuria was only documented in rats exposed to high dose of TNF-α for 5 days in Garin study [55]. Authors postulated that proteinuria might be the result of a direct effect on the glomerulus since the cytokine was infused directly into the renal artery rather on the effect of systemic TNF-α based on two facts: (1) there was no correlation between serum TNF-α and proteinuria and (2) absence of proteinuria in rats receiving 10 ng/h of TNF-α despite similar TNF-α serum levels than those observed in rats infused 20 ng/h.

Circulating TNF-α in Focal Segmental Glomerulosclerosis

Suranyi et al. [72] measured the TNF-α concentration in plasma of adult patients with MCD (n = 5), primary FSGS (n = 17), and membranous nephropathy (n = 12). There was a significant increase in TNF-α plasma levels in FSGS nephrotic patients compared to controls. Authors suggested a possible role of TNF-α in the development of proteinuria. However, this role seems unlikely because (1) 6 out of 17 patients with active FSGS had plasma levels of TNF-α similar to controls, and (2) plasma TNF-α levels did not correlate with proteinuria.

Anti-TNF-α Therapy

The beneficial role of anti-TNF-α therapy in proteinuria of the nephrotic syndrome has been suggested by case reports [73–76]. Only one of the four reported cases had FSGS as underlying disease [76]. The underlying disease was unknown in 1 patient [73], and MCD and amyloidosis associated to proliferative glomerulonephritis in the other 2 patients [74, 75]. Moreover, none of these patients received anti-TNF-α as single therapy, so the effect of concurrent immunosuppressive therapy cannot be excluded. The response to anti-TNF-α therapy was variable. While 1 patient remained with significant proteinuria despite anti-TNF-α therapy [73], 2 patients achieved resolution of proteinuria. One of them was started on anti-TNF-α by the time he had reached partial remission [75]. The other patient (MCD) achieved remission about 1 year after the first anti-TNF-α infusion [74]. However, patient's serum's TNF-α levels were much higher throughout the course of anti-TNF-α therapy than that observed prior to anti-TNF-α trial, and to those previously

reported in active MCD and FSGS patients, despite the resolution of proteinuria. This finding argues against a role of TNF-α as circulating factor causing proteinuria.

A successful response to anti-TNF-α therapy has been reported in one child who experienced FSGS recurrence after transplantation [76]. Unfortunately, serum TNF-α was not measured so it is unclear if there was a correlation between serum TNF-α and proteinuria. Moreover, the contribution of anti-TNF-α in this patient cannot be defined given the concomitant use of three immunosuppressive agents.

A randomized controlled trial including patients with active primary FSGS resistant to glucocorticoids plus another immunosuppressive agent (mycophenolate mofetil, cyclosporine, or tacrolimus) was designed to test the efficacy of anti-TNF-α (adalimumab) [77]. Preliminary results showed no significant changes in proteinuria or serum albumin in 10 patients after receiving adalimumab (24 mg/m^2 subcutaneously) every 14 days for 16 weeks (except for one patient who received 12 weeks). Unfortunately, no follow-up results have been published since 2010.

10.3.5.4 Transforming Growth Factor-β (TGF-β)

Systemic TGF-β

Kopp [78] developed transgenic mice that overexpressed TGF-β exclusively in the liver. Some of these transgenic mice demonstrated elevated plasma TGF-β levels and glomerular changes (mesangial expansion, deposition of immunoglobulins, and subendothelial deposits) at 3 weeks of age, prior to the onset of massive proteinuria at 5 weeks. Those mice with very high plasma TGF-β (>60 ng/ml) died of renal disease by 12 weeks of age. Authors suggested a role of systemic TGF-β in renal fibrosis.

Plasma TGF-β levels have not been well established in FSGS patients. Cheong [61] reported similar plasma TGF-β levels in patients with active MCD compared to active FSGS and in patients with idiopathic nephrotic syndrome in relapse (MCD and FSGS) compared to those in remission. Unfortunately, no control subjects were included in this study. Tain [79] found elevated serum TGF-β levels in children with idiopathic nephrotic syndrome in relapse compared to controls. The significance of this study is unclear since the underlying disease was not specified. In contrast, Goumenos [80] found similar plasma TGF-β levels in patients with glomerular diseases (including 5 FSGS patients) and controls. The absence of renal disease in other conditions associated with elevated TGF-β such as cancer makes uncertain the role of systemic TGF-β in renal fibrosis.

Urinary TGF-β and Proteinuria

Urinary TGF-β levels have been investigated in patients with FSGS and other glomerulopathies with conflicting results. Kanai [81] demonstrated increased

urinary TGF-β levels in 8 FSGS patients compared to controls, whereas patients with lupus nephritis, membranous nephropathy, and IgA nephropathy presented urinary TGF-β levels similar to controls. In contrast, Murakami [82] reported increased urinary TGF-β levels in 8 patients with FSGS but also in 13 patients with IgA compared to controls. Similarly, Goumenos [80] found elevated urinary TGF-β levels in patients with heavy proteinuria regardless the underlying disease (including five FSGS) compared to normal controls and IgA nephropathy without proteinuria.

The correlation between urinary TGF-β levels and proteinuria has shown also contrasting results. In Kanai's study, there was no correlation between urinary TGF-β and proteinuria in 33 patients with various glomerulopathies. Only 3 of 8 FSGS patients had proteinuria > 1 g/dl. Two FSGS patients had urinary TGF-β within the normal range. In contrast, Wasilewska [83] found that urinary TGF-β levels were increased in 24 patients with active FSGS compared to controls and correlated them with the degree of proteinuria. Goumenos [80] also observed a positive correlation between urinary TGF-β levels and proteinuria in patients with heavy proteinuria (including 5 FSGS patients) compared to controls or patients with IgA nephropathy without proteinuria. However, authors did not analyze such correlation according to the underlying disease.

Unfortunately, these studies did not provide a detailed clinical description of each patient with glomerular disease to better establish a relationship between urinary TGF-β and patients' underlying glomerulopathy. None of these studies, except for Goumenos' study, measured plasma TGF-β levels. In the majority of studies, urinary TGF-β correlated with proteinuria and with the severity of histological findings (mainly mesangial expansion) independent of the underlying disease. These observations suggest a role of TGF-β as local (intrarenal) mediator of kidney disease regardless the underlying glomerular findings.

10.4 Role of TGF-β in the Pathogenesis of Fibrosis in FSGS

FSGS is currently thought to be a podocytopathy [84]. Podocytes respond to injury by altering their morphology resulting in podocyte foot processes effacement which may lead to podocyte detachment from the GBM if the noxa persists. The detachment of podocytes has two critical consequences: (1) hypertrophy of surrounding podocytes that may result in further podocyte detachment and (2) bulging of the naked GBM which comes close to the parietal cells of Bowman's capsule leading to a parietal "beachhead" on the tuft initiating the process of fibrosis [85–87]. Glomerular lesions are irreversible at this point in time. Furthermore, this sequence of events may explain the segmental nature of the disease observed until, at least, advanced stages of renal disease. Massive proteinuria results in podocyte dysfunction and loss.

The role of TGF in the podocyte injury will be reviewed.

10.4.1 TGF-β and Renal Fibrosis

The transforming growth factor-β family includes TGF-β, activins, and bone morphogenic proteins. The majority of human cells have the capability to produce TGF-β and express receptors for it. TGF-β, synthesized as inactive form, requires cleavage by plasmin or thrombospondin-1 from the latent TGF-β binding protein (LTBP) or latency-associated peptide (LAP) to become active [88, 89]. TGF-β plays a key role as mediator of fibrosis as shown in multiple animal models [78, 90, 91] and in many human diseases [92–94].

10.4.2 Renal TGF-β

10.4.2.1 Animal Models of Fibrosis

There is solid evidence supporting a role of TGF-β in animal models of glomerulonephritis. In addition, TGF-β has been also shown to play a role in other animal models of renal disease such as diabetic nephropathy, HIV nephropathy, and ureteral obstruction model [95–97]. This is not surprising since glomerulosclerosis is a histological finding commonly observed in advanced stages of renal disease regardless of the underlying disease.

In an experimental model of acute glomerulonephritis, Yamamoto [98] found that rats infused with one dose of anti-mesangial serum (AMS) transiently overexpressed TGF-β in glomeruli associated with mesangial proliferation and proteinuria. A second dose of AMS (1 week after the first one) perpetuated glomerular TGF-β expression and proteinuria progressing to renal failure, glomerulosclerosis, and interstitial fibrosis with massive deposition of collagens by the week 18.

The in vivo transfection of TGF-β transgenic gene into normal rat kidneys led to increased production of TGF-β in glomeruli, rapid development of glomerulosclerosis, and proteinuria compared to control rats [99].

Border [91, 100] demonstrated that administration of anti-TGF-β antibody or decorin (proteoglycan that neutralizes TGF-β) concurrent with the induction of acute glomerulonephritis in rats prevented the increased production of matrix proteins by the glomeruli and blocked the accumulation of matrix.

10.4.2.2 Renal TGF-β Expression in FSGS

Yamamoto [101] observed an intense glomerular and tubular interstitial immunoreactivity (both intracellular and associated to matrix) for TGF-β in kidney specimens of 5 patients with FSGS compared to controls and patients with MCD but similar to other glomerular diseases such as IgA nephropathy, lupus nephritis,

diabetic nephropathy, and crescentic glomerulonephritis. Strehlau [102] demonstrated intrarenal TGF-β gene expression in 18 out of 20 patients with active FSGS but only in 3 out of 14 patients with MCD and IgM nephropathy. The majority of FSGS patients were on cyclosporine, known to promote TGF-β expression. Authors stated that immunosuppressive therapy was unlikely to be responsible of the difference in TGF-β expression since MCD patients were on steroids, which also increase TGF-β expression. Kim [103] performed immunohistochemical and in situ hybridization studies to determine the podocyte TGF-β expression in 15 FSGS patients. By immunohistochemistry, non-sclerotic FSGS glomeruli and glomeruli from normal controls showed no TGF-β or TGF-bIIR immunoreactivity. In FSGS, enhanced TGF-β signal was observed in areas of glomerulosclerosis. By in situ hybridization, signal for TGF-β mRNA was more intense, and not limited to sclerotic segments, in FSGS compared to controls. The signal was present in glomerular epithelial cells, mesangial, tubular, and endothelial cells. Goumenos [80] reported TGF-β expression, by immunohistochemistry, in the renal tissue of patients with heavy proteinuria including five with FSGS. Glomerular TGF-β expression was found only in patients with mesangial proliferation.

10.4.3 Anti-TGF-β Therapy

In 2011, an open-label study was design to determine in adults with active primary FSGS resistant to steroid or other immunosuppressive agents the safety, tolerability, and pharmacokinetic of a single dose of fresolimumab, a human monoclonal antibody, which blocks the three isoforms of TGF-β [104]. Preliminary results from 16 patients, who received different doses of anti-TGF-β therapy ($n = 4$ per group) and observed for 120 days, showed the drug to be well tolerated. As a secondary endpoint, data on kidney function and proteinuria were collected. Proteinuria fluctuated throughout the 120-day follow-up period. The median baseline protein-to-creatinine ratio (6.5 mg/mg creatinine) decreased by 1.1 mg/mg creatinine by the end of follow-up. There was also a slight decline in renal function with no dose-related differences. Conclusions cannot be drawn from this study since these data represent only preliminary data and a small number of patients.

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